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How to cite:

Silva, Ayona Therese; Wardhaugh, Tina; Dolatshad, Nazanin Fatima; Jones, Sarahlouise and Saffrey, Margaret Jill (2008). Neural progenitors from isolated postnatal rat myenteric ganglia: expansion as neurospheres and differentiation in vitro. *Brain Research*, 1218 pp. 47–53.

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Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.1016/j.brainres.2008.04.051>

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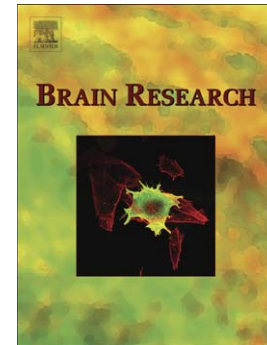
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PII: S0006-8993(08)00979-7
DOI: doi: [10.1016/j.brainres.2008.04.051](https://doi.org/10.1016/j.brainres.2008.04.051)
Reference: BRES 38038

To appear in: *Brain Research*

Accepted date: 15 April 2008



Please cite this article as: Ayona Therese Silva, Tina Wardhaugh, Nazanin Fatima Dolatshad, Sarahlouse Jones, Margaret Jill Saffrey, Neural progenitors from isolated postnatal rat myenteric ganglia: Expansion as neurospheres and differentiation *in vitro*, *Brain Research* (2008), doi: [10.1016/j.brainres.2008.04.051](https://doi.org/10.1016/j.brainres.2008.04.051)

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**Neural progenitors from isolated postnatal rat myenteric ganglia:
expansion as neurospheres and differentiation *in vitro***

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***Abbreviated title:* Neural progenitors from isolated postnatal
myenteric ganglia**

21 Pages text, plus 5 Figures, 0 Tables

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Abstract

Identification of the stem cell niche is crucial for understanding the factors that regulate these cells. Rodent enteric neural crest-derived stem cells have previously been isolated by flow cytometry and culture of cell suspensions from the outer smooth muscle layers or the entire gut wall from postnatal and adult animals. Such cell suspensions contain a mixture of cell types, including smooth muscle, fibroblasts and cells associated with the vasculature and extrinsic innervation. Thus these preparations may be contaminated by stem cells associated with extrinsic sensory and autonomic nerves and by other types of stem cell that reside in the gut. Here we describe a different approach, similar to that recently used for infant human gut, to obtain enteric *ganglion-derived* cells, with properties of neural progenitor cells, using isolated myenteric ganglia from postnatal rat ileum. Myenteric ganglia were separated from the gut wall, dispersed and resulting cell dissociates were plated in non-adherent culture conditions with EGF and FGF-2. Under these conditions neurosphere-like bodies (NLB) developed. Cells in NLB incorporated BrdU and expressed the stem cell marker nestin but not the pan-neuronal marker PGP 9.5. Upon growth factor withdrawal some BrdU-immunopositive cells assumed the morphology of neurons and expressed PGP 9.5; others were flattened and expressed the glial cell marker GFAP. This work therefore provides evidence that neural crest-derived progenitors in the postnatal rat gut are located in the myenteric plexus, and shows that these cells can be expanded and differentiated in NLB *in vitro*.

Classification terms:

Section 2: Nervous System Development, Regeneration and Aging

Keywords:

stem cells, neurogenesis, enteric nervous system, intestine, EGF, FGF-2, differentiation, proliferation

1. Introduction

Enteric neuronal precursors from the foetal gut have been studied by several authors, notably by Gershon and his co-workers, who have used immunoselection with antibodies to the low-affinity nerve growth factor receptor p75 (Chalazonitis et al. 1998a; Chalazonitis et al. 1998b) and to NC-1 (Pomeranz et al. 1993) to isolate neural crest-derived cells from the foetal rat gut. Recent studies however, have provided evidence that a population of neural crest-derived stem cells exists after birth, in the postnatal and adult rodent (Bondurand et al. 2003; Kruger et al. 2002) and human (Rauch et al. 2006a) (Almond et al. 2007) gut. To date, several different methods have been used to identify and select these cells from postnatal and adult rodent intestine. The first published method involves prospective flow cytometry of cell suspensions derived from outer smooth muscle layers (muscularis externa), in which myenteric ganglia are embedded; cells that express high levels of the low-affinity nerve growth factor receptor, p75 are selected (Kruger et al. 2002). A second, more frequently used approach is to grow dissociates of the outer muscle layers in conditions that favour the formation of neurosphere-like bodies (NLB, (Almond et al. 2007; Bondurand et al. 2003; Schafer et al. 2003)). The presence of stem cells in clumps of cells obtained from partial dispersal of the entire gut wall has also been inferred after such clumps were grown in adherent culture and gave rise to a range of cell types; however, no attempt was made to separate or enrich neural progenitors (Suarez-Rodriguez and Belkind-Gerson

2004). In all these studies, stem cells (or putative stem cells) were identified in mixed cell suspensions or cultures derived from different tissue types and different locations across the width of the gut wall (including in some cases, mucosal epithelial tissue, as well as muscular tissue). In addition to the intrinsic enteric nervous system, the gut is innervated by extrinsic autonomic and sensory nerves, and the possibility that some of the stem cells identified in these earlier studies were derived from crest-derived cells associated with these extrinsic nerve fibres cannot be ruled out. Since reliable markers for neural stem cells are not currently available, the location of enteric neural crest-derived stem cells within the rodent gut wall has not yet been established. Although it might be expected that they would reside within, or in close association with, enteric ganglia, to date no direct evidence for this hypothesis has been presented for the rodent gut.

Myenteric ganglia can be separated from the muscularis externa of the rodent gut, after a combination of enzyme treatment and mechanical agitation of the muscularis externa (Saffrey 1998; Schafer et al. 1997). The isolated ganglia may then be dispersed and cultured by standard methods (e.g. (Saffrey et al. 2000)). This technique has recently been used to isolate cells that give rise to NLB from the infant human gut (Rauch et al. 2006a). In the present study, we therefore sought to determine if neural progenitors could be obtained from isolated myenteric ganglia from the postnatal rat small intestine. The isolated ganglia were dissociated and the resulting cell suspension was maintained in non-adherent culture in the presence of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2), conditions that have previously been used to

generate CNS neurospheres (e.g.(Gritti et al. 1996; Reynolds and Weiss 1996)) and NLB from muscularis externa suspensions (Bondurand et al. 2003)..

Nestin is an intermediate filament protein that is expressed by neuroepithelial stem cells, and also by reactive astrocytes. It is used as a marker to analyse neural precursors in the developing CNS (Frederiksen and McKay 1988; Lendahl et al. 1990) and has been identified in crest-derived cells in the fetal rodent gut (Chalazonitis et al. 1998a). We therefore also examined if nestin is expressed in the postnatal enteric nervous system (ENS), and if it can be used as a marker for isolated neural progenitor cells.

We report that nestin mRNA is expressed in isolated enteric ganglia from postnatal rats, and that neural progenitors can be isolated from a cell suspension of isolated myenteric ganglia of postnatal rodents. These precursors are nestin immuno-positive *in vitro*, can be expanded as NLB under the influence of EGF and FGF-2, and are induced to differentiate into neurons and glial cells upon factor withdrawal. This work thus provides the first evidence that, as in humans, rodent enteric neural stem cells are located in the enteric ganglia.

2. Results

Separation of myenteric plexus from the non-neuronal tissues of the intestine

The myenteric plexus is embedded within the smooth muscle layers of the gut wall. In order to study the properties of myenteric ganglion cells in isolation from surrounding non-neuronal tissues, segments of plexus consisting of linked ganglia were separated by a combination of enzymatic digestion and mechanical agitation of the outer layers of the gut wall. The isolated segments of plexus (Figure 1 a) were harvested, inspected using a microscope and rinsed several times before use for RNA extraction or dissociation and cell culture.

Expression of nestin in the postnatal intestine and enteric ganglia

In order to investigate the possible use of nestin as a marker for undifferentiated neural precursors in the postnatal ENS, nestin expression was studied by RT-PCR. RNA was isolated from ganglia that had been separated by enzyme treatment from the non-neural tissue of the ileum of postnatal day 7 (n= 4 separate preparations) and 14 rat pups (n= 3 separate preparations; Figure 1c). Nestin expression was high in myenteric ganglia from both 7 and 14 day-old animals but negative in liver from the same animals (Figure 1b).

Expansion of enteric neural progenitors in vitro as spheres

To investigate whether neural progenitors could be obtained from isolated postnatal enteric ganglia, and expanded *in vitro* in a similar manner to CNS precursors, myenteric ganglion cells were isolated from both day 7 (n=7 separate isolations) and 14 rats (n=3 isolations), dissociated and plated at a density of 10^4 cells per 13mm coverslip (coverslip area = 133mm^2) in 24 well plates in the presence of EGF and FGF-2 (20ng/ml), without any exposure to serum or a synthetic adhesive substrate. Under these conditions, the great

majority of the dissociated enteric ganglion cells died but after 2 - 3 days, the very few cells that remained had proliferated and formed spheres, which were not attached to the coverslip, but which floated in the culture medium (Fig. 2a - d). In most wells, between 20 - 30 spheres typically arose; approximately 30% of wells had less than 10 spheres per well. The spheres increased in size when grown in the continuous presence of EGF and FGF-2 (Fig 2a-d) and their appearance under phase contrast optics was similar to that of neurospheres isolated from the CNS (Uchida et al. 2000). Cells isolated from enteric ganglia and grown in the presence of EGF and FGF-2 were maintained for up to 1 month as expanding spheres (the longest period studied). Incubation of spheres in BrdU and subsequent immunolabelling (n= 6 separate preparations of NLBs) confirmed that the cells were proliferating (Figure 3a). Immunolabelling (n= 3 separate preparations of NLBs) also demonstrated that the cells within the spheres expressed nestin (Fig.3b), but did not express the neuronal marker PGP 9.5 (data not shown). Although quantification of total cell numbers within NLBs was not possible, all visible cells were nestin- immunoreactive, and none were PGP 9.5 immunoreactive. No difference was seen between spheres obtained from 7 and 14 day postnatal animals. These data suggest that *in-vitro* culture conditions can be established for the prolonged proliferation of neural progenitors derived from the myenteric ganglia from postnatal rat pups.

Proliferation and differentiation of enteric neural precursors *in vitro*

In order to determine if the proliferating cells of the spheres were able to differentiate, EGF and FGF-2 were removed from the medium and the spheres

were plated on to an adhesive substrate (n= 6 for NLBs derived from 7 day postnatal rats; n= 3 for NLBs derived from 14 day postnatal rats). This treatment resulted in attachment and subsequent migration of two different types of cells from the spheres. One type of cell exhibited a typical neuronal morphology and the other a glial cell-like morphology (Fig. 4a).

The developmental potential of the proliferative cells in the spheres was then examined further. Nuclei of dividing cells were labelled after 5 days *in vitro* by 18hr exposure to BrdU (10 μ M). Double immunolabelling performed 3 days after removal of the mitogens showed a population of cells that were positive for both BrdU and the neuronal marker PGP 9.5 (from n=3 separate preparations of NLBs) (Fig. 4b and 4c). Cells that were immunopositive for both BrdU and the glial marker GFAP were also detected (from n=3 separate preparations of NLBs) (Fig. 5a and b). Some other cells that expressed these markers were BrdU immunonegative, indicating that they had withdrawn from the cell cycle before addition of BrdU on day 5 *in vitro* (Figures 4 and 5). The presence of BrdU immunopositive cells that also expressed either PGP9.5 or GFAP indicates that proliferating precursors *in vitro* were able to give rise to cells with the morphological and chemical phenotype of neurons or glia. Overall, these results indicate that a population of neural precursors with proliferative capacity exists in myenteric ganglia of postnatal rats, at least at 7 and 14 days of life.

3. Discussion

The results described here provide the first demonstration that neural progenitors can be obtained from isolated ganglia of the postnatal rat ENS and induced to proliferate as NLB *in vitro* under the influence of EGF and FGF-2. Differentiation of these progenitor cells was stimulated upon factor withdrawal and provision of an adhesive substrate. Detailed quantification of the different types of cells within the NLBs, and of the different types of cell that differentiate upon factor withdrawal was not possible in this study, but such analysis would form an important part of future work. Whether these progenitor cells are members of the same population of neural stem cells previously identified by flow cytometry of the muscularis externa (Kruger et al. 2002) remains to be established. Similar methods have been used to generate neurospheres from isolated human enteric ganglia (Rauch et al. 2006a). The distribution and the abundance of these cells in the myenteric plexus remain to be determined. It is likely, however, that they only represent a small population of cells in the postnatal ENS.

A difficulty in the study of neural stem cells, and indeed of restricted or partially restricted precursors, is the lack of markers that unambiguously label a single cell type, or cells at a single stage of their development (see e.g. (Svendsen et al. 2001; Young et al. 2003). Kruger et al (2002) used high level of p75 to select neural crest stem cells from the dissociated muscularis externa. However, only 1-2 % of cells that express high levels of p75 formed multi-lineage colonies in culture (Kruger et al. 2002) and differentiated enteric glial cells also express p75

(Young et al. 2003), so this marker cannot be used in intact gut preps (sections or whole mounts) to identify neural crest-derived stem cells. In the CNS, uncommitted neural precursor cells and committed neuroblasts and glioblasts express nestin, which is down-regulated in postmitotic neurons (Frederiksen and McKay 1988), but up-regulated in astrocytes after some injuries (Julien 1999). In the ENS, evidence suggests that nestin expression is lost by precursors that are committed, but still proliferating (Chalazonitis et al. 1998a). Although nestin-immunoreactive cells have been detected in the human foetal gut (Rauch et al. 2006b; Vanderwinden et al. 2002), nestin-immunopositive cells in intact gut preparations from postnatal rodents have not yet been identified, possibly because they represent only a very small population of cells. The expression of nestin by cells of intact myenteric ganglia demonstrated in the present study by RT-PCR is nevertheless indicative of the existence of a population of uncommitted neural progenitors in the myenteric plexus of postnatal rats during the first two weeks of postnatal life.

Indirect evidence for the localisation of neural precursors within the myenteric ganglia of postnatal mice has also come from the work of Young et al. (2003) who identified a small population of cells in the postnatal myenteric plexus that do not express either neuronal or glial markers (PGP 9.5 or S100, respectively). 1.7 % of cells in the myenteric plexus of day-old postnatal mouse ileum and 4.7% in the colon did not express either marker. This small population of cells decreased in number with increasing postnatal age, and constituted only 0.3% of cells in the myenteric plexus of the adult ileum, although labelling was described as uncertain in a slightly larger population. Since these cells did not

express markers of embryonic enteric neural crest-derived stem cells (e.g. Ret or Phox2b), the authors have pointed out that these cells have a different phenotype to embryonic enteric crest-derived stem cells and may therefore represent a different type of cell. This does not rule out the possibility that they are neural precursor cells and are able to proliferate and form NLBs under appropriate circumstances.

The evidence described here and by others for the existence of myenteric plexus-derived precursor cells in the early postnatal gut is in keeping with morphological and immunohistochemical studies that have demonstrated that the ENS undergoes a number of changes postnatally (Fausone-Pellegrini et al. 1996; Matini et al. 1997; Schafer et al. 1999; Vannucchi and Fausone-Pellegrini 1996). It has also been established that some enteric neurons withdraw from the cell cycle postnatally (Pham et al. 1991). In the present study, no difference in the properties of the NLBs and derived cells from 7 and 14 day postnatal animals was seen, but postnatal changes between 7 and 14 days cannot be ruled out. More detailed studies, using additional neuronal markers, may reveal postnatal changes in the numbers or properties of these cells.

The continued presence of a population of neural precursor/stem cells within the gut into adulthood (Bondurand et al. 2003; Kruger et al. 2002) suggests that these cells may be important in ENS plasticity, and could be involved in gastrointestinal disorders which involve the ENS. Plasticity in the ENS is likely to continue into maturity, as this part of the nervous system is subject to complex physical and molecular changes due to its location and interactions

with a diversity of cell types within the gut. Evidence from surgical interventions (Galligan et al. 1989) and chemical removal of areas of the myenteric plexus (Luck et al. 1993) (Hanani et al. 2003) show clear evidence for regeneration of enteric nerve fibres and for differentiation of immature neurons. Enteric neural precursors may normally be quiescent, but may be stimulated to proliferate and differentiate in response to stimuli such as injury. In inflammatory bowel disease, for example, as well as neurodegeneration, an increase in neuronal numbers in the adult ENS has been described (see (Lomax et al. 2005; Vasina et al. 2006). The ENS is a unique part of the nervous system in that it is potentially highly vulnerable to damage by exposure to high levels of harmful toxins and inflammatory mediators, particularly if the mucosal epithelium is damaged. Therefore some regeneration of neurons in the ENS may occur in adult animals, and as in the adult brain, neurogenic potential in the ENS may persist throughout life.

Neuronal degeneration and dysfunction are implicated in many intestinal disorders, particularly neuromuscular disorders, which affect gastrointestinal motility. Recent work has shown that neural stem cells from other sources grafted into the stomach can integrate into the ENS and restore some function (Micci et al. 2005a; Micci et al. 2001; Micci et al. 2005b), see (Micci and Pasricha 2007). Understanding the biology of resident enteric neural stem cells is therefore important, to optimize the outcome of such potentially valuable therapeutic approaches.

In conclusion, this work provides evidence that enteric neural-crest derived stem cells reside in the enteric ganglia, and that isolated enteric ganglia can be used as a source of neural crest-derived cells that can be expanded as neurospheres *in vitro*.

4. Experimental Procedure

Isolation and dissociation of myenteric ganglia; generation of neurosphere-like bodies

Sprague-Dawley rat pups (7d and 14d old) were killed by cervical dislocation (Schedule 1, approved by the British Home Office and Open University Animal Ethical Committee) and intestines were removed. The ileum was used to obtain myenteric plexus as previously described (Saffrey 1998; Schafer et al. 1997). Briefly, the muscularis externa was removed from the ileum by peeling under a dissecting microscope and rinsed twice in Hanks Balanced Salt Solution (HBSS) containing gentamycin (200 µg/ml) and metronidazole (50 µg/ml). The tissue was cut into 2-3 mm strips and incubated in collagenase (1mg/ml, CLSII, Worthington Laboratories, USA) supplemented with DNase (10 µg/ml). After a brief vortex, clean pieces of myenteric plexus that were completely freed from smooth muscle were harvested, rinsed twice and dissociated using trypsin/EDTA. Cells were plated at a density of 1×10^4 /13 mm glass coverslip without any substrate or adhesive factors, in serum-free medium (199; Sigma, with added N1 supplements), containing EGF (human recombinant, Sigma) and FGF-2 (human recombinant, Sigma) at 20 ng/ml. These conditions were designed to minimise attachment and survival of differentiated cells, but to

promote proliferation of neural precursors and were modified from published methods used to expand CNS precursors as neurospheres *in vitro* (Gritti et al. 1996; Reynolds and Weiss 1996) .

Analysis of cultures

NLB and their constituent cells were studied in several ways:

1. Live cultures were examined and photographed using phase contrast optics.
2. At various times after they had formed, NLB were transferred onto poly-L-lysine (PLL)-coated coverslips for a short time to allow attachment (but not flattening), then fixed for 1hr in 4% paraformaldehyde in phosphate buffered saline, and then immunolabelled as described below.
3. To investigate the differentiation potential of cells growing in spheres, the NLB were re-seeded, without EGF and FGF-2, onto PLL-treated coverslips to initiate adhesion. This procedure was performed at various time-points, and cultures were subsequently maintained for periods of up to 3 d. The resulting adherent cultures were then fixed as described above.
4. To study proliferation of cells in the NLB, bromodeoxyuridine (BrdU, 10 μ M) was added to cultures on day 5, for a period of 18 hrs before removal of mitogens and transfer to PLL-treated coverslips, as described in 3, above. BrdU-treated cultures were grown for 3 d post-adhesion before fixation as in 2, above.

Immunolabelling and cell imaging

Neurons were identified using an antiserum raised against ubiquitin hydrolase (also known as protein gene product 9.5, PGP 9.5, rabbit anti-human, Ultraclone), or β -III tubulin (Promega). Immunopositive cells were revealed using a biotinylated secondary antibody, followed by streptavidin fluorescein (Amersham, Life Science). Enteric glial cells were identified using a monoclonal anti-GFAP antibody (Novocastra). To identify nuclei that had incorporated BrdU, fixed cells were pre-treated with 2N HCl for 25min, followed by 0.1M sodium borate for 25 min and then incubated in a mouse monoclonal anti-BrdU antibody (Becton Dickinson). Positive nuclei were visualised using Texas-red conjugated horse anti-mouse IgG (Vector Laboratories). Nestin immunostaining was carried out using a mouse monoclonal anti-nestin antibody, Rat-401 (Hybridoma Bank, University of Iowa, USA) and revealed using a Texas-red conjugated anti-mouse IgG. Immunostained cell preparations were imaged using a Leica TCS-NT confocal microscope.

RT-PCR

RNA from isolated myenteric ganglia (n=4 different preparations of isolated ganglia) and liver was extracted using a method modified from that of (Chomczynski and Saachi 1987). Tissues were disrupted by homogenising in solution D (4 M guanidine thiocyanate, 25 mM sodium acetate, 0.5% sarcosyl, 1% 2-mercaptoethanol) and phenol chloroform extraction. First strand cDNA was synthesised with 21 μ g of total RNA, random hexamers and Superscript II reverse transcriptase, in a volume of 40 μ l. cDNA (1 μ l) was then amplified with

primers for nestin or β -actin in a PTC-100 PCR with Taq polymerase in a final volume of 50 μ l. Nestin primers were 5' -CTA GGG TCT GTG GAT GAG AAC and 3' -AGC TTC TTC CTC TCC ACC TTG and were based on rat nestin from embryonic CNS designed to amplify a 330 bp fragment of DNA (Genebank accession No. M34384). β -actin primers were 5' -TCA TGA AGT GTG ACG TTG ACA TCC and 3' -CCT AGA AGC ATT TGC GGT GCA CGA and were designed to amplify a 285 bp fragment (Promega, UK).

Acknowledgements:

This work was supported by the Open University Research School.

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Figure Legends:**Figure 1:**

a) Myenteric ganglia separated from the muscularis externa of 7 d rat ileum. Scale bar 1.5mm. b) RT-PCR showing nestin expression in the myenteric plexus isolated from the ileum of 7 d (lane 2) and 14 d (lane 3) rat pups, but not in liver (lane 4).

Figure 2: EGF and FGF-2 induce development of neurospheres from ENS cells in culture.

Neurosphere-like clusters develop when dissociates of myenteric ganglia are grown in the absence of serum, synthetic substrate or adhesive factors, in the presence of EGF and FGF-2 (20 ng/ml). Spheres develop by day 3 (a) and expand in size; the same sphere is shown at (b) 5days, (c) 10 days and (d) 2 weeks in vitro. Scale bar 20 μ m.

Figure 3: Cells in myenteric plexus-derived NLB proliferate and express nestin

Immunolabelling shows (a) a population of cells in a neurosphere incorporate BrdU, and (b) cells in a 5 d neurosphere are nestin positive. Scale bar (a) 40 μ m (b) 25 μ m

Figure 4: Growth factor withdrawal from ENS-derived neurospheres results in differentiation of neurons from proliferating precursors.

Expanded neurospheres from myenteric plexus of 7 d pups, incubated in BrdU for 18hr and then cultured on poly-lysine coated coverslips after withdrawal of EGF and FGF-2. (a) Some precursors undergo differentiation into neurons, identified by immunolabelling with anti-PGP 9.5 (green cells). (b) Some of the PGP9.5 immunopositive neurons are BrdU immunopositive (red nuclei), (c) composite image showing BrdU positive, PGP 9.5 positive neurons (yellow nuclei), indicating that they had arisen from a precursor cell that has previously proliferated in culture. Also note that some non-neuronal cells (possibly glia) ηαπε ρεδ νυχλει. Σχαλε βαρ 12 μm .

Figure 5: GFAP immunopositive glia differentiate from proliferating precursors in NLB in vitro.

(a) Some precursors undergo differentiation into GFAP immunopositive glial cells (green). (b) some of the GFAPpositive cells are BrdU positive nuclei, indicating that they were proliferating while in spheres, prior to growth factor removal. Scale bar 20 μm .

Figure 1

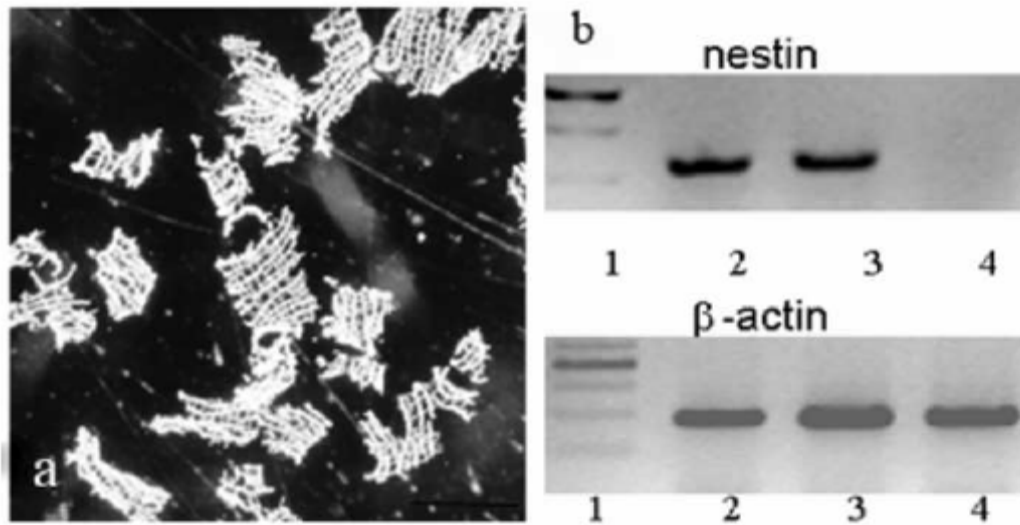


Figure 2

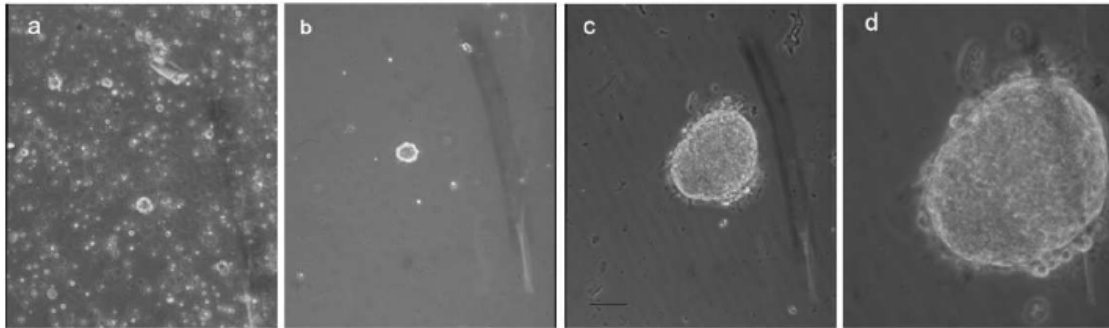


Figure 3

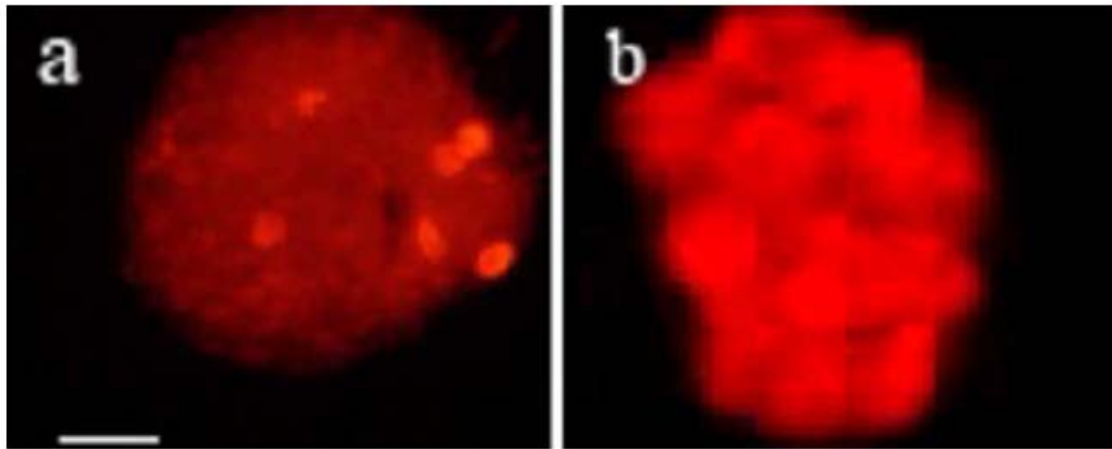


Figure 4

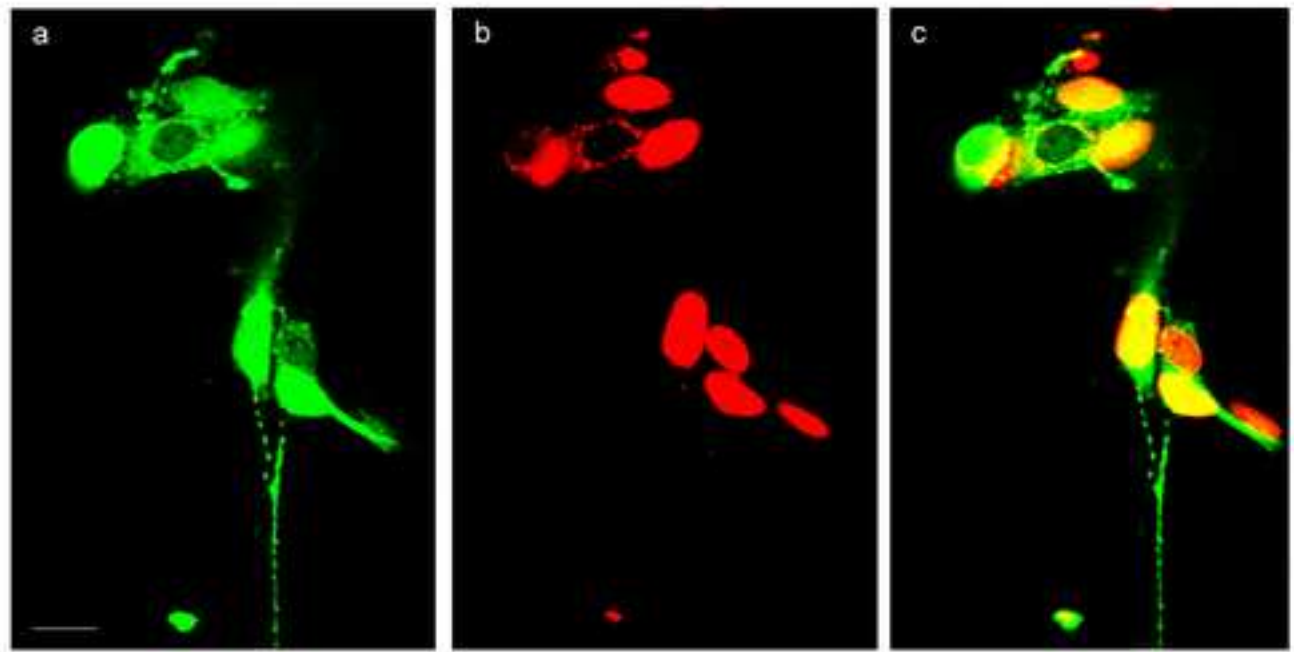


Figure 5

